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14. ABSTRACT The sea mussel *Mytilus edulis* adheres tenaciously to surfaces that exhibit a wide range of properties in seawater. A study was conducted to gain a better understanding of the interactions between two of the mussel foot proteins found in the adhesive plaque (Mefp-1 and Mefp-2) and several substrata with different surface properties (germanium, polystyrene and poly (octadecyl methacrylate). Mefp-1 excludes Mefp-2 from a germanium surface until the L-dopa residues of Mefp-1 are converted to the quinone form, suggesting that Mefp-1 is a bi-functional primer that requires activation by a catechol oxidase. However, Mefp-2 adsorbs to germanium, polystyrene and poly (octadecyl methacrylate) at least as strongly as Mefp-1, indicating that Mefp-1 does not possess unique properties that allows it to out-perform other mussel foot proteins in forming an adhesive bond with the substratum. We also tested the hypothesis that nucleophilic addition of the ε-amino groups of lysine residues into the oxidized catechol functionality (quinone) of the L-dopa residues in Mefp-1 is a cross-linking reaction in the plaque. Experimental results did not support this hypothesis. The plaque-forming reactions responsible for mussel adhesion to surfaces remain elusive.

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Characterization of Molecular Interactions of *Mytilus edulis* Foot Proteins on Model Hydrated Surfaces

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Publications Resulting from Project

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Students Theses Resulting from Project

Bryan Bowie, Ph.D. dissertation, "A Study of Factors Affecting the Performance of Infrared and Raman Spectrometers", Department of Chemistry, University of Idaho, Moscow, ID, December, 1999.

Project Summary

The sea mussel *Mytilus edulis* has become a model organism for investigation of adhesion. Studies have been inspired by the sea mussel's ability to form a tenacious attachment to surfaces with a wide range of chemistries in a seawater environment within minutes. From an engineering perspective, the sea mussel offers design possibilities for a biomimetic glue that sets underwater. Investigation of the mechanism of mussel adhesion to a submerged substratum has been facilitated by a relatively advanced understanding of the biochemistry of the various components that condense to form the adhesive plaque.

The adhesive plaque of *M. edulis* is composed of a mixture of biopolymers that have associated to form a number of condensed phases. *M. edulis* foot protein Mefp-1 is the most well-characterized component of the sea mussels adhesive plaque. Though originally thought to be the primary adhesive, Mefp-1 is now known to account for approximately 5% of the plaque protein and is present at the plaque substratum interface as well as forming a protective "varnish" along the top surface of the attachment plaque and along the outer surface of the byssus (Rzepecki et al, 1992). While Mefp-1 clearly plays a structural role in the byssal thread, its role in adhesion is now less clear (Vreeland et al, 1998).

Mefp-1 is densely populated with lysine and L-3,4-dihydroxyphenylalanine (L-dopa) residues incorporated into a repeating amino acid sequence motif (Waite et al, 1985). It has been postulated that Mefp-1 participates in mussel adhesion to a substratum through oxidation of the catechol moieties to the corresponding quinone via a catechol oxidase enzymatic reaction (Waite, J.H., 1987). In an attempt to confirm that this mechanism is viable, infrared spectroelectrochemical measurements were developed and carried out in two ways.

In the first approach, the modulated beam from a rapid-scanning interferometer was passed through a thin layer of electrolyte containing the electroactive analyte and reflected from the surface of a platinum electrode. The beam passed back though the electrolyte and was focused on the detector. We successfully observed the oxidation of several small molecules containing the dihydroxybenzene moiety, including hydroquinone, l-dopa and epinephrine. In each case, the product of the electrochemical oxidation was different, indicating a mechanism that depended on the structure of the catechol. Only for hydroquinone was the oxidation reversible. Regrettably, oxidation of Mefp-1 did not yield interpretable results.

In an attempt to improve the surface selectivity of the spectroelectrochemical measurements, we attempted to invoke the use of the surface-enhanced infrared absorption (SEIRA) effect by coating the surface of an internal reflection element (IRE) with an island coating silver or gold. It was known from previous work in Griffiths' and other laboratories that the absorption of infrared radiation by species within 5 nm of the thin vapor-deposited Au or Ag surfaces is enhanced by between one and two orders of

magnitude. For the spectroelectrochemical experiments, a thick (150 nm) layer of gold was vapor deposited around the edge of the IRE to allow good electrical contact and a thin (~5 nm) layer of the metal was deposited on the center of the IRE. This layer was thin enough that the evanescent wave would pass through the layer with relatively minor attenuation (about 20-30% per pass), so that an adequate signal could be observed at the infrared detector. Initially, a germanium (Ge) IRE was used in light of the fact that Ge is electrically conductive (albeit with quite high resistance). However, even for hydroquinone, for which the redox cycle is usually reversible, the electrochemistry proved to be very intractable. Ultimately it was found that electrochemical corrosion of the Ge surface seriously affected the performance of this cell. We then switched to a zinc selenide (ZnSe) IRE and proved that it was possible to perform reversible redox cycles on gold-coated ZnSe IREs. We believe that the cell that was designed and fabricated right before the end of this project will allow the oxidation of the mussel adhesive proteins to be followed but have not quite reached the point at which these experiments can be initiated.

During this project, we found that the shape of the absorption bands that are measured during SEIRA experiments is dependent on the thickness of the Au or Ag layer and the nature of the IRE. Slight differences were also found between transmission and ATR measurements of the identical sample. This effect has great significance on the interpretation of the spectroelectrochemical SEIRA experiments.

If there exists a specialized primer for the mussel adhesion reaction, it must not only adsorb rapidly and tenaciously to surfaces having a variety of properties, but must also induce binding of components that are structural members of the adhesive plaque. The primer molecule must therefore exhibit bi-functionality, having functional groups that bind to the surface and others that are free to bind to other plaque components. One plaque cross-linking reaction that has been proposed is the nucleophilic addition of the ε-amino groups of the lysine residues into the oxidized catechol functionality (quinone) of the L-dopa residues (Waite, J.H., 1987). An objective of the project was to acquire data to support this cross-linking reaction in Mefp-1.

A previously developed assay for ε-amino groups was employed to determine whether this reaction occurs in adlayers of Mefp-1 (Suci and Geesey, 2000). Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was used to characterize these interactions at a germanium (Ge) interface. Adlayers were exposed to an oxidant; either the enzyme mushroom tyrosinase or sodium periodate. Binding of alginate was used to probe for accessibility of ε-amino groups. It was found that lysine residues lost the ability to bind alginate after exposure to sodium periodate, but that this loss is not clearly due to a reaction with L-dopa residues. The apparent reaction of sodium periodate with amines was unanticipated. There was a slight decrease of binding of alginate to adlayers of Mefp-1 exposed to either active or thermally inactivated mushroom tyrosinase, perhaps due to obstruction of binding sites by bound enzyme. The kinetics of adsorption of mushroom tyrosinase onto adlayers of Mefp-1 for active and thermally inactivated enzyme was nearly identical. The study suggests that while exposure of adlayers of Mefp-1 on Ge to sodium periodate may induce formation of aryl-amines, exposure to mushroom tyrosinase does not. These data do not support the hypothesis that cross-linking involves lysine ε-amino group addition to the quinone functionality of oxidized L-dopa in Mefp-1. Future research should consider the role of

the catechol oxidase as a cross-linking agent in the mussel plaque. It has also been suggested that another protein in the Mefp family (Mefp-3) has a "molecular asymmetry" that is consistent with the bi-functional role of primer (Warner and Waite, 1999).

The patterns of order exhibited by the primary structures of the Mefp family of proteins suggests that the proteins have specialized roles in a self-assembly process that results in the well-bonded, highly condensed adhesive plaque. A second objective of the project, therefore, was to identify specialized functions among the different foot proteins produced by *M. edulis*. To this end, a series of experiments were conducted to compare surface binding of Mefp-1 and Mefp-2. Mefp-2 contributes about 25% of the plaque protein content, is about a third the size of Mefp-1, and is highly cross-linked via cystine disulfide bonds (Rzepecki et al, 1992). It has been proposed that Mefp-2 is a structural component of the plaque, whereas Mefp-1 mediates surface bonding (Rzepecki et al, 1992). On the basis of these proposed functions, we hypothesized that Mefp-1 should exhibit faster adsorption kinetics and stronger interactions with a substratum than Mefp-2. ATR-FTIR spectroscopy was used to follow the kinetics of appearance of proteins at the surface of a Ge internal reflection element (IRE) mounted in a flow cell designed to introduce proteins in solution to the interfacial region. The Ge substratum was exposed to a solution of Mefp-1 for 60 min, then the surface rinsed for 30 min. Following the rinse period, the Ge substratum was then exposed to a solution of Mefp-2 for 60 min, and the surface rinsed again for 30 min. The laminar flow cell design provides controlled hydrodynamics allowing investigation of the effect of diffusion on kinetics of adsorption by regulating the flow rate. The influence of different catalysts on the interfacial interactions can also be investigated.

Difference spectra were used to follow the interaction of solution phase Mefp-2 with the preadsorbed Mefp-1 adlayer. The kinetics of protein adsorption to the surface were fit with an equation describing the sum of two first order formation rates

$$K + C_1 (1-\exp(k_1 t)) + C_2 (1-\exp(k_2 t)) \qquad (1)$$

where t is time, Estimates of K, C_1, C_2, k_1, and k_2, were found by a least squares fit criterion. The floating parameter, K releases the constraint that the predicted curve pass through the origin, thereby decreasing the influence of entrance kinetics on delivery of protein to the interface.

The results suggest that Mefp-2 adsorbs to the Ge substratum at least as strongly as Mefp-1 (Suci and Geesey, 2001a). Thus, Mefp-1 does not appear to possess any unique properties that allow it out-perform other mussel foot proteins in forming an adhesive bond with the substratum as originally proposed. Both the surface coverage, measured at the end of the rinse period and the surface coverage predicted for infinite adsorption time by the fit to the equation ($\Gamma = C_1 + C_2 + K$) indicate that the presence of a preadsorbed, non-oxidized, adlayer of Mefp-1 excludes Mefp-2 from the interfacial region (Suci and Geesey, 2001a). Exposure of the Mefp-1 adlayer to the oxidant, sodium periodate, increases surface coverage of Mefp-2 to levels comparable to those for Mefp-2 adsorption onto a clean Ge substratum. These results show that Mefp-1 excludes Mefp-2 from the surface until the L-dopa residues are converted to the quinone form. This suggests that Mefp-1 serves as a bifunctional primer that requires activation by the

catechol oxidase. This is speculative, however, since the results suggest that Mefp-2 adsorbs to the Ge substratum at least as strongly as Mefp-1.

The above experiments were repeated using substrata with different surface properties. The behavior of the proteins were compared on polystyrene (PS) with aromatic character, poly(octadecyl methacrylate) (POMA) with aliphatic character and Ge with oxide character. Since the estimates of error for the rate constant (k_2) and surface coverage (Γ) for the slow component of the adsorption process were sometimes larger than the mean, and the error estimates for the rate constant (k_1) and surface coverage (Γ_1) for the more rapid component of the adsorption process are relatively reliable (S.E., 10% mean), the latter were used for the comparison of protein adsorption on the different surfaces. The importance of the slower process was evaluated, however.

The main conclusion drawn from the results of the study is that there are no pronounced differences between either the rate of adsorption or the surface coverage attained over short (<60 min) time periods for Mefp-1 and Mefp-2 (Suci and Geesey, 2001b). According to these criteria Mefp-1 and Mefp-2 are ranked about equal in terms of being a putative agent (primer) that mediates bonding to the substratum. In fact, despite speculation that Mefp-1 has special adhesive properties, by every measure except long term adsorption (Γ), Mefp-2 adsorbs faster and with greater surface coverage than Mefp-1.

The similarity in adsorption behavior of Mefp-1 and Mefp-2 onto surfaces having quite different properties was unexpected. Although both proteins have L-dopa residues and both contain oligopeptide repeats, they are otherwise quite different. The most pronounced difference between the kinetic data is the lower surface coverage of Mefp-1 on POMA. One possible explanation is that specific interactions between the lysine and/or L-dopa residues of Mefp-1 and the Ge and PS surfaces (absent for the POMA surface) contribute to the binding site density (Baty et al, 1997). Frank and Belfort (2001) have recently shown that the attractive binding energy during separation of Mefp-1 from substrata with different surface properties varied depending on whether the substratum critical surface tension (γ_c) was greater than or less than that of Mefp-1. They also reported unusually close predictions between the Johnson, Kendall and Roberts theory and experimental data. By performing direct measurement of adhesion and characterizing the protein adsorption behavior, a more complete understanding of the protein-surface interactions can be obtained.

The methodology developed in this project can be used to further characterize interactions between the Mefp family of proteins on a surface. The influence of a catalyst such as catechol oxidase can be tested. The ability of family members to induce adsorption of other plaque components can be probed by this approach. It may also be possible to use distinctive spectral features of each Mefp family member, as we have done with Mefp1 and Mefp-2, to determine relative densities of Mefp proteins in mixed adlayers, thus making it possible to do competition studies without labeling the proteins.

Another objective of the project was to determine the properties of a surface containing adlayers of the mussel proteins. To this end, the critical surface tension (γ_c) was determined for adlayers of Mefp-1 and Mefp-2 on mica. Mica was used as the primary substratum for γ_c determination because of its molecular smoothness, thus alleviating the influence of surface roughness on contact angle measurements. Mefp-1 and Mefp-2 were prepared as aqueous solutions at 0.1 mg/ml in pH 8.5 phosphate buffer,

exposed to the surfaces for 24h at 4C, rinsed to remove unadsorbed protein and dried *in vacuo* for 2 h at 22C. The surface energy of the adsorbed proteins was determined using 9 liquids which span a wide range of surface tensions. Drops of the liquids were placed on the dried protein adlayer and the resulting contact angle measured. The cosine of the contact angle was plotted against the surface tension of each of the 9 test liquids. Where the best fit linear regression of the data intercepts the axis where $\cos \theta = 0$, the critical surface tension γ_s is obtained according to Eqn 2.

$$\lim_{\cos \theta \to 1} \gamma = \gamma_c \qquad (2)$$

Based on this approach, $\gamma_{c,s}$ for Mefp-1 and Mefp-2 was 37.5±2.7mN/m and 40.0±3.1 mN/m, respectively. Since these values are higher than the $\gamma_{c,s}$ value for a bare mica surface (27.3± 3.7 mN/m), the protein adlayers appeared to cover the surface. The value for Mefp-1 fell within the range of globular proteins whose $\gamma_{c,s}$ values were determined under the same conditions (bovine serum albumin, 35.2±2.8 to 37.1±1.1 mN/m; g-globulin, 39.7±2.7 mN/m). Olivieri et al (1990) reported $\gamma_{c,Mefp\text{-}1}$ values of 32.9±0.9 mN/m for layers of Mefp-1 spread on a Ge substrate. The $\gamma_{c,s}$ obtained for Mefp-1 is higher than expected, but no explanation for this result is available at this time.

In summary, the *Mytilus edulis* foot proteins Mefp-1 and Mefp2 alter the properties of the surfaces to which they adsorb. Mefp-1 excludes Mefp-2 from a surface until Mefp-1 L-dopa residues are oxidized. Mefp-2 adsorbs to germanium, polystyrene and poly (octadecyl methacrylate) at least as strongly as Mefp-1, indicating that Mefp-1 does not possess unique properties that allows it to out-perform other mussel foot proteins in forming an adhesive bond with the substratum. Nucleophilic addition of the ε-amino groups of lysine residues into the oxidized catechol functionality (quinone) of the L-dopa residues in Mefp-1 does not appear to be the cross-linking reaction in the plaque. The plaque-forming reactions responsible for mussel adhesion to surfaces remain elusive.

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